EVALUATION OF VARIOUS MEDIA AND ANTIMICROBIAL AGENTS FOR ISOLATION OF SOIL FUNGI

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During the last 30 years considerable progress has been made toward the development and standardization of culture substrata for the isolation, enumeration, and identification of soil fungi. Many of these substrata have been largely ineffective in inhibiting growth of the more abundant bacteria and streptomycetes isolated from soils, and in eliminating the "masking" effect of rapidly growing and often-coalescing fungal colonies of such genera as *Mucor*, *Rhizopus*, and *Trichoderma*. To circumvent these commonly experienced difficulties, workers in the field of soil microbiology have used various antimicrobial agents. The development of antibiotics has made it possible, through their inclusion in culture media, to improve conditions for fungi by eliminating certain classes of bacteria from the plates (1, 3, 5, 7, 9). Acid has often been added to the media to favor the growth of fungi (16, 17). Other agents such as crystal violet and rose bengal also have proved effective (8, 9, 11, 14). During the last decade the use of oxgall (oxbile) (1, 8, 10, 11), sodium desoxycholate (5, 15), and sodium propionate (4) has also been suggested. The literature dealing with these agents has been adequately summarized in several papers (1, 3, 9, 10) and need not be repeated here.

The objectives of the present investigation were (*a*) to screen, under comparable conditions, some of the media used in microbiological laboratories and several media not suggested previously for the isolation of soil fungi, so that the better media could be selected for detailed studies; and (*b*) to select one or more media that would give maximum fungal counts on dilution plates, would eliminate bacteria, and would facilitate counting and identification of a wide range of fungal genera while simultaneously restricting the development of rapidly growing fungal colonies.

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MATERIALS AND METHODS

From 41 media screened in preliminary tests, 34 were eliminated on the basis of several undesirable points, mainly low number of colonies per gram of soil, and failure to eliminate bacteria or restrict colony size of rapidly growing species of fungi. The following media were selected for further studies:

(*a*) Modified Warcup's medium. Warcup's medium (18) plus 5.0 g. oxgall, 1.0 g. sodium propionate, and 35 mg. chlortetracycline per liter (7); pH 6.3.

(*b*) Dextrose-peptone-yeast extract agar (DPYA). Dextrose, 5.0 g.; peptone, 1.0 g.; yeast extract, 2.0 g.; NH_4NO_3, 1.0 g.; K_2HPO_4, 1.0 g.; $MgSO_4 \cdot 7H_2O$, 0.5 g.; $FeCl_3 \cdot 6H_2O$, trace; oxgall, 5.0 g.; sodium propionate, 1.0 g.; agar, 20.0 g.; distilled water to make 1 liter. Sterilization at 11 pounds for 15 minutes. Add 30 mg. of chlortetracycline and 30 mg. of streptomycin per liter.

(*c*) V-8 juice[2]-dextrose-yeast extract agar (VDYA). V-8 juice, 200 ml.; distilled water, 800 ml.; $CaCO_3$, 3.0 g. (12). Bring to boiling; filter through cotton and cheesecloth. Agar, 20.0 g.; dextrose, 5.0 g.; yeast extract, 2.0 g.; oxgall, 5.0 g.; sodium propionate, 1.0 g.; distilled water to make 1 liter. Sterilization at 11 pounds for 15 minutes. Add 30 mg. of chlortetracycline and 30 mg. of streptomycin per liter.

(*d*) Modified VDYA. VDYA plus 2.0 g. of peptone per liter.

(*e*) Ohio medium (OAES) (4).

(*f*) Modified Martin's medium. Martin's medium (9) plus 5.0 g. oxgall and 1.0 g. sodium propionate per liter.

(*g*) Modified Raper's medium. Raper's medium (13) plus 5.0 g. oxgall, 1.0 g. sodium propionate, 35 mg. chlortetracycline, and 30 mg. chloromycetin per liter.

Results on acidified glucose-peptone agar (17)

[2] Trademark of a vegetable-juice product of the Campbell Soup Company.

TABLE 1

Fungal colonies on soil-dilution plates from 5 different soils on 8 media

Medium	Average Number of Colonies per Plate* from Indicated Soil					
	Greenhouse loamy sand	Composted Elsinboro sandy loam	Elsinboro sandy loam	Russellville silt loam	Wheeling silt loam	Average per medium
Modified Warcup's	43.4 *ab*†	38.4 *ab*	32.8 *a*	44.5 *a*	26.4 *a*	37.1 *a*
DPYA	50.5 *a*	42.0 *a*	30.5 *a*	41.4 *a*	19.8 *b*	36.8 *a*
Modified VDYA	42.8 *b*	40.6 *a*	30.2 *a*	46.4 *ab*	21.8 *a*	36.4 *a*
VDYA	42.5 *b*	39.2 *a*	30.0 *a*	44.4 *a*	21.6 *a*	35.5 *a*
Ohio medium	40.5 *b*	32.1 *bc*	25.6 *b*	33.8 *c*	14.9 *bc*	29.4 *b*
Modified Martin's	42.9 *b*	35.2 *abc*	13.6 *c*	36.5 *bc*	13.1 *c*	28.3 *b*
Modified Raper's	40.8 *b*	29.9 *c*	22.2 *b*	31.9 *c*	12.5 *c*	27.5 *b*
Acidified glucose-peptone agar	24.1 *c*	21.8 *d*	14.4 *c*	22.6 *d*	6.2 *d*	17.8 *c*
L.S.D. (0.05)	7.2	6.2	6.2	7.2	5.2	4.2
L.S.D. (0.01)	9.6	8.3	8.3	9.6	7.0	5.6

* Average of 8 replications.

† Numbers followed by the same italic letter are not significanty different from each other at the 5 per cent level of probability by Duncan's multiple range test (6).

were used as the standard against which to compare other results throughout the experiments.

Soil dilutions were prepared from greenhouse loamy sand, composted Elsinboro sandy loam, Elsinboro sandy loam, Russellville silt loam, and Wheeling silt loam as follows: the equivalent of 5.0 g. oven-dry soil was suspended in 500 ml. sterile tap water and blended in a Waring blendor for 1 minute at approximately 2500 rpm. Five replications of each final dilution were prepared and pooled prior to plating. One-ml. aliquots were transferred to each of eight petri dishes for each medium.

All the antimicrobial agents except the antibiotics[3] were incorporated with the original ingredients of the media. Fresh solutions of antibiotics were added after the media had been melted and cooled to approximately 48°C. Plates were incubated at 25°C. and colonies were counted after 2, 3, and 4 days. Fungal genera were identified after 10 days.

RESULTS

Comparison of selected media

The experimental results of a series of tests comparing the eight selected media at a dilution of 1–5000 prepared from five different soils are summarized in table 1. Considering the overall means of five soils, there were three levels of significance by the Duncan's multiple range test (6). Modified Warcup's medium, DPYA, modified VDYA, and VDYA all allowed significantly more colonies of fungi to develop on the dilution plates than did Ohio, modified Martin's, or modified Raper's medium. Acidified glucose-peptone agar (pH 4.0) allowed the smallest number of colonies to develop.

The overall analysis of variance indicated significant interaction between media and soils at the 1 per cent level. This finding is in agreement with observations that experience with one soil and a particular medium does not necessarily hold good for other soils (2). Individual analyses were carried out to evaluate the media with each particular soil. Although such analyses resulted in some changes in the relative position of media shown in table 1, modified Warcup's, DPYA, modified VDYA, and VDYA maintained their superiority to the other four media.

Incorporation of antimicrobial agents (antibiotics, oxgall, and sodium propionate) in the appropriate media listed in table 1 reduced the size of spreading fungal colonies so that counts and isolations of relatively slow-growing species of fungi were feasible. From the standpoint of total number of genera and facility of identification on

[3] Chloromycetin was furnished by Parke, Davis & Company; chlortetracycline (aureomycin) by Lederle Laboratories.

the initial dilution plates, DPYA and VDYA were superior to the other media (table 2). No interference from bacteria or streptomycetes was observed on the media tested.

Effect of oxgall and sodium propionate on the numbers of fungi isolated from soil

Two factorial experiments involving three levels of oxgall and sodium propionate were carried out in order to determine the optimum concentration of these antimicrobial agents, alone or in combination, that would allow high numbers of fungal colonies to develop on the dilution plates with as little interference from bacteria and fast-spreading colonies of fungi as possible. DPYA as described under MATERIALS AND METHODS but without oxgall and sodium propionate was used as basal medium in the first experiment, and VDYA without these agents in the second. In addition to the nine media included in table 3, DPYA and VDYA as originally described were included in both experiments for comparison.

In both experiments the overall response to oxgall and to sodium propionate *per se* was statistically significant at the 1 per cent level of probability but their interaction was not. The response to sodium propionate, which was linear, was unfavorable in regard to numbers of fungi per gram of oven-dry soil produced in both experiments. The response to oxgall, on the other hand, which had both linear and quadratic characteristics (table 3), was favorable in both basal media. In the experiment with VDYA, the linear component of the effect attributed to oxgall had significance at the 1 per cent level of probability, while the quadratic component was significant at the 5 per cent level. In the experiment with DPYA these levels of significance were reversed. These results demonstrate that, with VDYA and increasing concentrations of oxgall up to 5.0 g. per liter, the number of colonies per plate increases, though the rate of increase is greater between 0 and 2.5 than between 2.5 and 5.0 g. per liter. With DPYA, however, the quadratic characteristic of the response to oxgall was more pronounced, as indicated by the higher number of colonies obtained with 2.5 g. of oxgall per liter of medium. This finding is in agreement with the observation that the effectiveness of oxgall and other agents is conditioned by the nature of the plating medium (11).

The VDYA comparison treatment allowed significantly higher counts of fungi per plate than the average of the nine media within the factorial design (113,000 fungi per g. of oven-dry soil). The DPYA comparison treatment was identical with the average of the other nine media (86,000 fungi per g. of oven-dry soil). However, the color, size, and texture of the individual colonies on both

TABLE 2

Genera of each class of fungi isolated by the dilution-plate method from 4 soils on 8 media*

Medium	Phycomycetes	Ascomycetes	Basidiomycetes	Fungi Imperfecti	Total
VDYA	7	1	1	30	39
DPYA	8	1	1	28	38
Ohio medium	8	1	0	24	33
Modified VDYA	6	1	0	23	30
Modified Raper's	8	1	0	21	30
Modified Warcup's	7	0	0	22	29
Acidified glucose-peptone agar	8	0	1	15	24
Modified Martin's	5	0	0	17	22

* Greenhouse loamy sand, Elsinboro sandy loam, Russellville silt loam, and Wheeling silt loam.

TABLE 3

Effect of varying concentrations of oxgall and sodium propionate in DPYA and VDYA on the numbers of fungi enumerated by the dilution-plate method from Elsinboro sandy loam

Basal Medium and Concentrations of Sodium Propionate	Number of Fungi* for Three Concentrations of Oxgall			
	0.0 g./l.	2.5 g./l.	5.0 g./l.	Total
g./liter				
DPYA:				
0.0	74	127	104	305
2.5	71	95	81	247
5.0	64	89	74	222
Total	209	311	259	
VDYA:				
0.0	65	115	106	286
2.5	53	88	101	242
5.0	55	71	76	202
Total	173	274	283	

* Thousands of fungi per gram of oven-dry soil.

comparison media were superior for ease of enumeration and identification to those of colonies on the other nine media. Despite the fact that the same concentration of antibiotics was used in all media, DPYA plus 2.5 g. of oxgall per liter, which gave the highest number of fungal colonies, was also characterized by a high number of bacterial colonies per plate. Evidently 5.0 g. of oxgall per liter plus the antibiotics is more effective in eliminating bacteria than 2.5 g. of oxgall per liter.

Effect of oxgall, rose bengal, sodium desoxycholate, and sodium propionate on the numbers of fungi isolated from soil

The sixteen media evaluated in table 4 included the basal medium DPYA as described but without oxgall and sodium propionate. The four antimicrobial agents were added to the basal medium either singly or in combination at the following concentrations per liter: Oxgall, 5.0 g.; rose bengal, 0.033 g.; sodium desoxycholate, 0.3 g.; sodium propionate, 1.0 g.

When a soil dilution of 1–5000 was employed, the various agents used singly or in combinations did not affect the number of fungal colonies per plate (table 4). Evidently when the number of fungal colonies per plate is relatively low, and the degree of interference among developing colonies through competition and antagonism is not serious, the effectiveness of antimicrobial agents may not be appreciable. When all platings were done from a 1–2000 dilution of the same soil, highly significant differences among the various agents and combinations of agents in their effect on counts of soil fungi were observed. Oxgall and sodium desoxycholate, when added singly, allowed significantly higher counts of fungi per plate over those obtained on the basal medium or on media containing only rose bengal, or sodium propionate. The mean effect due to oxgall, sodium desoxycholate and the interactions OX + SD and OX + RB were significant at the 1 per cent level, whereas the interactions SD + SP, OX + RB + SD, and OX + RB + SD + SP were significant only at the 5 per cent level of probability.

Although it is apparent that there is virtually no response to sodium propionate and no significant interaction between it and oxgall, the medium containing 5.0 g. of oxgall and 1.0 g. of sodium propionate per liter gave the highest arithmetic mean in class *a* of significance. This apparent beneficial effect on the total counts of fungi due to the combination of oxgall and sodium propionate persisted despite the verdict of non-significance.

An examination of the colonies after 10 days of incubation indicated that the proportion of fungal isolates that failed to sporulate on the original dilution plates was relatively low. Even on the medium with all four agents, only 16 per

TABLE 4

Effect of oxgall, rose bengal, sodium desoxycholate, and sodium propionate on the numbers of fungal colonies on soil-dilution plates from Elsinboro sandy loam

Agents* Added to Basal Medium (DPYA)	Dilution 1–5000 Colonies† per Plate	Dilution 1–2000	
		Colonies† per plate	Mean effect‡ and interactions
None (control)	30.4 *a*§	66.2 *d*	—
OX	31.1 *a*	114.6 *ab*	22.1**
RB	35.1 *a*	76.5 *cd*	—
SD	32.4 *a*	107.5 *b*	16.6**
SP	28.9 *a*	63.2 *d*	—
OX + RB	29.6 *a*	110.6 *b*	23.6**
OX + SD	30.9 *a*	119.2 *ab*	38.7**
OX + SP	33.2 *a*	126.4 *a*	—
RB + SD	27.8 *a*	116.6 *ab*	—
RB + SP	29.1 *a*	87.1 *c*	—
SD + SP	34.8 *a*	112.2 *ab*	16.6*
OX + RB + SD	30.0 *a*	113.2 *ab*	45.2*
OX + RB + SP	32.6 *a*	113.2 *ab*	—
OX + SD + SP	34.4 *a*	108.9 *b*	—
RB + SD + SP	30.0 *a*	106.5 *b*	—
OX + RB + SD + SP	29.9 *a*	106.5 *b*	40.3*
L.S.D. (0.05)	7.2	13.0	
L.S.D. (0.01)	9.5	17.2	

* Key to the agents added: OX, oxgall; RB, rose bengal; SD, sodium desoxycholate; SP, sodium propionate.

† Average of 8 replications.

‡ Mean effect of an agent is the mean of the difference between counts on all media containing the agent and all media from which the agent is absent (19). Only significant figures at the 5 (*) and 1 (**) per cent levels are listed.

§ Numbers followed by the same italic letter are not significantly different from each other at the 5 per cent level by Duncan's multiple range test.

cent of the fungal colonies were sterile after 10 days of incubation. Higher concentrations of these antimicrobial agents, however, seem to suppress sporulation (10).

DISCUSSION

From the results of experiments designed to find media useful for enumerating, identifying, and isolating soil fungi, it appears that modified Warcup's medium, dextrose-peptone-yeast extract agar (DPYA), and V-8 juice-dextrose-yeast extract agar (VDYA) in its original or a modified form, are all worth recommending. Because of significant interactions between soils and media, however, no single medium was best for all soils tested. Although modified Warcup's, the Ohio, modified Martin's, and modified Raper's media gave high colony counts, the number of genera identified on the original dilution plates was smaller than that on DPYA and VDYA.

None of these media is of a chemically exact nature since they contain agar, oxgall, peptone, V-8 juice, or yeast extract, which are of variable composition. The use of these ingredients cannot now be avoided without obvious detriment to the desirable characteristics of the medium. Dilution plates with media lacking oxgall or some other fungistatic and bacteriostatic agents become no longer advisable if they are to circumvent the crowding effect of rapidly growing fungal colonies and the undesirable interference resulting from bacterial colonies.

Many discussions can be found as to the effectiveness of various antimicrobial agents used singly or in various combinations. From the present experiments it appears that oxgall is the most effective agent in restricting colony size of fast-spreading fungi and thus facilitating enumeration and isolation of high numbers of fungal species from soil. Its effectiveness, nonetheless, seems to depend on the concentration used, on the composition of basal medium, and on the number of potential colony sites on the plates. Though only limited concentrations were used, it is evident from table 3 that the optimum, when used alone with basal DPYA and VDYA, was obtained at 2.5 g. per liter. This is considerably lower than the amounts used by others (1, 8, 10). When oxgall was used in combination with sodium desoxycholate or with sodium propionate (tables 3 and 4) the maximum number of colonies was obtained when 2.5 and 5.0 g. oxgall per liter were used with basal DPYA and VDYA, re spectively. When potential colony sites on plat are not crowded, there seems to be no advanta in using such antimicrobial agents other than reduce colony size, provided adequate conce trations of antibiotics are used to elimina bacterial growth. With lower soil dilutions co siderable improvement of the media can b attained by incorporating oxgall or various comb nations of oxgall and the other three agents.

In conclusion, the following points wer brought out by the present investigation: (a DPYA and VDYA appeared the most sati factory of 41 media compared for isolation of so fungi; (*b*) antibiotics and other agents such a oxgall are indispensable for making media suitabl for quantitative work; and (*c*) oxgall in excess o 0.25 to 0.5 per cent in media is of dubious valu

SUMMARY

From 41 media recommended for isolation o soil fungi, modified Warcup's medium, dextrose peptone-yeast extract agar (DPYA), V-8 juice dextrose-yeast extract agar (VDYA) and a modification of VDYA were the most valuable for isolating the greatest number of fungi from soil. In addition to the main ingredients, all these media contain two broad-spectrum antibiotics (chlortetracycline + streptomycin), oxgall, and sodium propionate or sodium desoxycholate. Ohio, modified Martin's, and modified Raper's media were also satisfactory for the isolation of high numbers of fungi from soil. DPYA and VDYA allowed appreciably higher numbers of fungal genera to be identified on the original dilution plates than the other five media evaluated.

Oxgall at 0.25 to 0.5 per cent of the medium was the most effective agent in diminishing the spreading of rapidly growing fungi and in allowing greater numbers of colonies to develop per plate though not appreciably reducing their sporulation. Various combinations of oxgall, rose bengal, sodium desoxycholate and sodium propionate were also effective in allowing high counts of fungi per plate and in reducing interference from spreading colonies.

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